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During the last year we have continued our investigation of the neurochemical systems contained in the circadian clock localized within the suprachiasmatic nucleus (SCN). Our primary focus has been to determine the circadian functions of a subpopulation of SCN interneurons in which vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI) and gastrin releasing peptide (GRP) have been co-localized. In these studies we have demonstrated: 1) VIP/PHI neurons are found in the ventrolateral SCN where photic projections terminate, 2) a rhythm of VIP/PHI mRNA within the SCN that correlates with the day-night cycle, 3) a modulation of SCN content of VIP and PHI by environmental lighting, 4) combined microinjection of VIP/PHI/GRP into the SCN mimics the phase delay of circadian rhythms produced by light, and 5) VIP/PHI/GRP alters the electrical activity of SCN single units. In other studies (con't)

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we have investigated the possible circadian functions of arginine vasopressin, GABA and neuropeptide Y (NPY) within the SCN, and the role of NPY within the paraventricular nucleus on corticosterone secretion.

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Circadian clocks serve to generate rhythms in mammalian behavior and physiology and then synchronize these rhythms with the 24 hr day-night cycle. The long-term goals of this project are to determine the neurochemical mechanisms within the suprachiasmatic nucleus (SCN) involved in these timekeeping functions.

Vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI) and gastrin releasing peptide (GRP) within the SCN

Day-night rhythms of VIP/PHI mRNA within the SCN

VIP and PHI are derived from a common polypeptide precursor. Using immunocytochemistry and in situ hybridization we have demonstrated that VIP and PHI immunoreactivity and mRNA are localized in close association with the photic projections terminating in the SCN. We subsequently demonstrated that light suppressed VIP and PHI immunoreactivity within the SCN, but did not influence the levels of neurotensin or substance P immunoreactivity. In the last year we have completed studies using quantitative in situ hybridization to examine the influence of environmental lighting on VIP/PHI gene expression within the SCN. Autoradiographs of the SCN from rats killed 5 hrs after the onset of the daily light period were compared to those of rats killed 2 hrs after the onset of darkness. Visual inspection of the autoradiographs from the SCN of the two groups of rats that were processed simultaneously under identical hybridization conditions revealed a striking day-night difference in hybridization signal. Grain density in the autoradiographs of rats killed during the dark period appeared to be much greater than in the rats killed during the light period. To more objectively compare the density of hybridization signal within the autoradiographs from the two groups a computer based image analysis system was employed. The SCN and adjacent control area were outlined on a reverse dark field image of each section in which the silver grains were not visible. The optical density was then calculated for the SCN and control region of the dark field autoradiograph on a pixel by pixel basis. The ratio of the SCN/control region grain density (which therefore controlled for background differences) was found to be significantly ($P<0.05$) greater in rats killed at night than during the day.

While quantitative in situ hybridizataion indicates whether differences exist in mRNA levels, it does not provide a reliable estimate of the absolute amount of these differences. As a result a second experiment was conducted to confirm the day-night variation in VIP/PHI mRNA and determine the amount of the day-night difference in the cellular levels of VIP/PHI mRNA with solution hybridization. Rats were again maintained in a 14:10 light-dark cycle and killed either 5 hrs after lights on or 2 hrs after darkness. The brains were frozen on dry ice and a 500 micron punch was used to remove the SCN and a small region of the cortex adjacent to the midline from the brain of each rat. Solution hybridization revealed that VIP/PHI mRNA was 2-3 fold greater ($P<0.01$) in the SCN of rats killed during the dark than in rats killed during the day. Furthermore, the day-night rhythm in VIP/PHI mRNA appeared to be restricted to the SCN since no signficant day-night differences were observed in the cortex of these same animals.

Effects of VIP, PHI and GRP microinjected into the SCN on the timing of circadian rhythms in vivo

It has recently been reported that most of the VIP/PHI immunoreactive neurons within the SCN also contain a third biologically active peptide, gastrin releasing peptide (GRP). To examine the functional role of these peptides in circadian control a cocktail of the three peptides (VIP/PHI/GRP) in a 1:1:1 molar ratio were microinjected into the SCN (N=39). VIP/PHI/GRP microinjection produced statistically significant delay shifts of over 1.5 hr in the hamster circadian activity rhythm. Importantly, these phase delays were observed only when VIP/PHI/GRP was microinjected during the phase of the circadian cycle when light also produces phase delay shifts. At all other phases of the circadian cycle VIP/PHI/GRP microinjection has little or no effect on circadian timing. These data therefore suggest that VIP/PHI/GRP containing SCN interneurons may be involved in the processing of environmental lighting information within the SCN. We are currently examining the effects of microinjecting each peptide alone and in combination with one of the other two peptides to begin to investigate the functional significance of their co-localization.

Effects of VIP, PHI and GRP on SCN single unit electrical activity in vitro using the hypothalamic slice preparation

To further characterize the role of VIP/PHI/GRP and the functional significance of their co-localization in SCN interneurons we have begun studies to identify the response of SCN neurons to each of the three peptides provided individually, and in combination with one or both of the two other peptides. VIP, PHI and GRP provided individually (10^{-7}) produce a small excitatory response in SCN single units. However when all three peptides are administered together (10^{-7}), the excitatory response is approximately four fold greater. Preliminary data indicate that adminstration of two of the three peptides produces an excitatory response intermediate to that seen following administration each peptide alone and that seen following adminstration of all three in combination. These experiments combined with the microinjection studies described above should clarify the functional significance of the co-localization of VIP/PHI/GRP within the SCN.

Arginine vasopressin (AVP) within the SCN

In vitro studies: Effects of AVP on circadian function within the SCN

Other investigators have demonstrated that AVP mRNA content, AVP concentration and AVP release within the SCN occur in a circadian pattern that peaks during the light period, suggesting that AVP could be involved in meditiating some of the effects of light within the SCN. To examine whether the release of AVP from SCN interneurons may be important in the control of circadian rhythms we determined how SCN single units respond to AVP.

Using the in vitro hypothalamic slice preparation, other laboratories had demonstrated that the single unit activity of SCN neurons exhibit circadian variations in rat SCN. However since the hamster is the species used for our in vivo studies because of the precise nature of its behavioral circadian rhythms we have studied SCN

neuronal activity in the hamster hypothalamic slice so these data can be compared with the in vivo studies. Our initial studies which characterized the spontaneous neuronal activity of hamster SCN have revealed that the spontaneous activity is very similiar to that previously reported in the rat. SCN neurons exhibit 3 patterns of spontanous firing and the discharge rate occurs in a circadian pattern with peak firing during the light period. We have also demonstrated that a rhythm of spontaneously firing can still be observed in the SCN of hamsters housed in constant light for at least 5 months.

After fully characterizing the spontaneous firing patterns in hamster SCN, we investigated the effects of AVP on SCN activity. AVP was found to have excitatory effects on 51% of the 74 SCN neurons examined. Since AVP release has been reported to peak during the light period, we examined whether a corresponding rhythm existed in the response of SCN neurons to AVP. Surprisingly, the response to AVP within the SCN was found to be rhythmic, however the peak response to AVP occurred during the dark phase of the LD cycle. While only 24% of SCN units responded to AVP during the light period, 73% responded to AVP during the dark phase. Further dose-response studies revealed that not only did fewer units respond during the light phase, but that those that did respond were less sensitive to AVP during the light phase than during the dark phase. In a next series of experiments we examined whether the effects of AVP in the SCN are mediated by a V1 or V2 AVP receptor. Using selective V1 and V2 antagonists and agonists the effects of AVP in the SCN were found to be mediated by a V1-like receptor. Following definition of the AVP receptor within the SCN it was possible to investigate the hypothesis that AVP contributes to the circadian rhythm of spontaneous discharge. In support of this hypothesis are the findings that the peak spontaneous discharge of SCN neurons and the peak in AVP release occur at the same time of day, and the finding that AVP can excite at least some SCN neurons. If AVP contributes to the excitatory peak in spontanteous discharge seen during the light period it should be possible reduce this peak of excitatory activity by inhibiting AVP activity. We tested this hypothesis using an AVP antagonist that was effective in blocking the excitatory effects of AVP within the SCN and found that inhibiting AVP activity had no effect on the spontaneous rhythm of single unit activity.

Neuropeptide Y (NPY) within the SCN and paraventricular nucleus (PVN)

Effects of NPY microinjected into the SCN and PVN on blood corticosterone levles

NPY immunoreactive terminals are found in both the SCN and PVN. We have previously demonstrated that NPY terminals within the SCN may be involved in communicating environmental lighting information to the circadian clock. In the last year we have completed studies examining whether NPY might also be involved in the regulation of adrenocorticoids by acting in either the SCN or PVN. Blood levels of corticosterone were determined in groups of rats that received microinjections of NPY or saline (SAL) into the PVN or SCN. NPY injected into the PVN 4 hrs after light onset resulted in corticosterone levels of 13.15+2.18 ug/dl within 1 hr, which were significantly higher than the corticosterone levels of 4.08+1.78 seen in rats receiving SAL injections. In contrast, no significant

differences were observed in circulating levels of corticosterone between groups of rats 1 or 4 hr after NPY or SAL microinjection into the SCN. These data indicate that NPY may participate in the regulation of adrenocorticoid secretion by acting on neurons within the paraventricular region of the hypothalamus.

Effects of NPY on single unit activity within the rat SCN and PVN

Since NPY appears to have functional significant effects within both the SCN and PVN we have begun studies to identify the response of SCN and PVN single units to NPY using the hypothalamic slice preparation. The spontaneous firing of the majority of neurons sampled in both the SCN (i.e. 32/29) and PVN (i.e. 24/31) was found to be altered by NPY. NPY had primarily inhibitory effects, however some units in both SCN and PVN displayed excitation. One difference in the response of SCN and PVN was that approximately 20% of SCN neurons displayed excitation followed by pronounced inhibition. The data collected to date suggest that NPY may shift circadian rhythms and elevate corticosterone as a result of inhibition of the activity of neurons within the SCN and PVN, respectively.

Effects of GABA and low chloride perfusate on SCN single unit activity

Since GABA appears to be contained in a subpopulation of SCN neurons and several studies suggest that manipulation of GABAergic activity may influence circadian rhythms we examined the effects of GABA on SCN single units. As expected, GABA was found to have a potent inhibitory effect on the discharge from SCN single units. No day-night differences were found in the percentage of units responding to GABA or in the dose-response sensitivity. In a related study the effects of reducing the concentration of chloride ions in the slice perfusate was examined. Exposure to low Cl- medium during the light phase (80% of NaCl was replaced with equimolar Na isethionate) abolished the activity in most neurons (12/14). In contrast, during the dark phase low Cl- medium excited most spontaneously firing neurons (9/11) and initiated activity within some previously silent neurons. These data suggest that chloride channels may be involved in the circadian rhythm of spontaneous discharge within the SCN.

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